UTILIZATION OF VARIOUS AGRO-INDUSTRIAL BY-PRODUCTS/WASTES AS SUBSTRATE BY RHAMNOLIPID PRODUCER Serratia rubidaea SNAU 02 UNDER SOLID STATE FERMENTATION

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Abstract

This study was aimed to identify the potentiality of a Rhamnolipid producer from Serratia rubidaea SNAU02 to utilize various agro industrial by-products/waste such as castor oil cake (CSOC), coconut oil cake (COC), gingelly oil cake (GOC), peanut oil cake (POC), mahua oil cake (MOC) and sunflower oil cake (SOC) as a substrate for the production of biosurfactant under solid state fermentation. Among the several agro-industrial by-products screened for the production of biosurfactant, mahua oil cake showed higher production of biosurfactant followed by peanut oil cake. Among the levels used, 8 g of mahua oil cake recorded the higher emulsification index, reduction of surface tension and biosurfactant production of 73 ± 0.86 %, 34 ± 0.21 mN/m and 81 ± 0.24 mg/g.ds, respectively. The peanut oil cake registered second best substrate of surface tension of emulsification index, surface tension and biosurfactant production of 69 ± 0.86 %, 35 ± 0.21 mN/m and 71 ± 0.24 mg/g.ds, respectively. The present findings indicated that mahua oil cake as suitable substrate for the production of Rhamnolipid under SSF by Serratia rubidaea SNAU02.

Introduction

Biosurfactants are biological surface active agents, produced by variety of microorganisms such as bacteria, fungi and yeast. Biosurfactants are classified by various methods – ionic nature, type and size (Desai and Banat, 1997). Biosurfactants have diverse chemical structures, compositions and a broad range of uses in various industries (Jain et al., 2012). The biosurfactant are surface active agents which includes lipopeptide, glycolipids, phospholipids and peptides. Biosurfactant can be produced on various substrates and has potentiality of replacing the chemical surfactants which cause environmental hazards. However, the price of raw materials usage significantly increases the cost for production of biosurfactant (Nalini et al., 2016). The submerged fermentation pose problem with foam formation during the production (Lee and Kim, 2004; Yeh et al., 2006), hence solid-state fermentation (SSF) could be an alternate method for the biosurfactant production. Solid state fermentation (SSF) has continued to built up credibility in recent years in biotech industries due

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to its potential applications in the production of biologically active secondary metabolites, apart from feed, fuel, food, industrial chemicals and pharmaceutical products and has emerged as an attractive alternative to submerged fermentation (Pandey, 2003; Singhania et al., 2009). There are very few reports on the production of biosurfactant by SSF (Kiran et al., 2010; Nalini and Parthasarathi, 2014). Microorganisms differ in their requirements for carbon sources, including quantities, as well as for other requisite micronutrients, for their metabolic activities. This makes it necessary to identify suitable agro-waste for each isolate that has shown a tendency for biosurfactant production on refined substrates. Advances in the utilization of agro-waste/agro-industrial by-products such as substrate for the production of biosurfactants have been on the rise, as more of these wastes are being identified as appropriate carbon and nitrogen sources (Sobrinho et al., 2013; Amodu et al., 2014).

Oil cakes and oil meals are by-products used after the oil extraction from the oil seeds. Depending upon the extraction methods the chemical composition of oil cake varies. These oil cakes are fairly rich in protein and are traditionally used as feed ingredients for farm animals. They are also used as aquaculture feeds (Singh et al., 2003). Oil cakes are of two types, edible and non-edible. Oil cakes have been in use for feed applications to poultry, fish and swine industry. Being rich in protein, some of these have also been considered ideal for food supplementation. However, with increasing emphasis on cost reduction of industrial processes and value addition to agro-industrial residues, oil cakes could be ideal source of proteinaceous nutrients and as support matrix for various biotechnological processes. Several oil cakes, in particular edible oil cakes, offer potential benefits when utilized as substrate for bioprocesses (Ramachandran et al., 2007). In the light of the above, present study aims to explore the feasibility of agro-industrial by-products/wastes as substrate for the production of biosurfactant by Serratia rubidaea SNAU02 employing SSF.

2. Materials and Method

Microorganism

S. rubidaea SNAU02 (Accession Number KC560769), a potent biosurfactant producer was used for the present study (Nalini and Parthasarathi, 2013). The strain was grown in nutrient agar (NA), sub-cultured each month and stored at 4 °C. The inoculum was prepared by transferring a loopful of cells into 25 ml Nutrient medium in 250 ml Erlenmeyer flask and incubated at 30 °C for 24 hrs.

Substrate preparation

Agro-industrial by-products such as peanut oil cake (POC), coconut oil cake (COC), gingelly oil cake (GOC), castor oil cake (CSOC), sunflower oil cake (SOC) and mahua oil cake (MOC) were used as substrate in the present study. The isolated strain was tested for biosurfactant production under solid state fermentation utilizing the effective agro industrial waste as substrates. All the substrates were obtained from the local market in Chidambaram, Tamil Nadu, India, dried at 60 °C for 48 hrs, blended to fine powder using a mixer-grinder, sieved using 1.0 mm sieve, packed in air-tight polythene bags and stored in moisture-free container at room temperature. Proximate analysis was carried out on the processed samples to determine the crude fibre, ash content, protein, phosphorous, potassium and carbohydrate using standard methods of analysis. Total nitrogen-Semi automatic Kjeldhal apparatus (Bremner, 1965). Total potassium - Neutralization triacid with ammonia and reading in Flame photometer (M/s. Elico, India) (Jackson, 1973). Total phosphorous – Vanadomolybdic Calorimetric method (Jackson, 1973). Total protein - Lowry’s method (Lowry et al., 1951). Total carbohydrates - The presence of carbohydrates of the substrates estimated by the method described by Chaplin and Kennedy (1994), Total fibre and Ash content - (AOAC, 1990). Each test was conducted in three replicates and average values are recorded.
Solid state fermentation

To study the effect of agro-industrial wastes as a substrate on biosurfactant production, the SSF flasks were supplemented with a mineral salt solution containing NH$_4$NO$_3$ - 0.5, NaCl - 0.9, MgSO$_4$ 7H$_2$O - 0.1, for 100 g dry substrate, initial pH and moisture of the substrates were set at 6.5-7.0 and 65 % respectively. SSF experiments were conducted in 250 ml Erlenmeyer flask each containing 4, 6, 8, 10 and 12 g of substrates individually such as peanut oil cake, coconut oil cake, gingelly oil cake, castor oil cake, sunflower oil cake and mahua oil cake. Then, the flasks containing substrates were sterilized at 121 °C for 15 min, cooled and inoculated with 2.0 ml inoculum of S. rubidaea SNAU02 and incubated at 30 °C for seven days.

Extraction of biosurfactant under solid state fermentation

The extraction of biosurfactant from SSF was performed by acid precipitation, followed by liquid – liquid extraction method. For this, 100 ml of distilled water was added to each SSF flask and then the contents were agitated at 200 rpm at 30 °C for 1 hour on an orbital shaker. Then, the contents were filtered using a cheese cloth and the excess of filtrate was squeezed out. This procedure was repeated for three times and the filtrates were pooled and followed by centrifugation at 12,000 rpm for 10 min. The supernatants were acidified using conc. HCl to pH 2.0 and then 100ml of 1:1 chloroform: methanol was added to each flask. The organic phase was separated and the biosurfactant was dried in a rotary vacuum evaporator. The extracted biosurfactant was stored in an air-tight container (Nalini et al., 2016).

Analytical method

Emulsification index (% EI$_{24}$)

The emulsification index (% EI$_{24}$) was used as a measure of the quantity of biosurfactant as described by Nitchke and Pastore (2004). Coconut oil was added to the cell-free supernatant in a ratio of 2:2 (v/v) and vortexed for 2 min. The percent ratio of the height of emulsified zone total height after 24hrs gave the emulsification index (%EI$_{24}$) following the equation as below:

\[
\% \text{EI}_{24} = \frac{\text{Height of emulsified zone}}{\text{Total height of liquid (sum of aqueous, oil and emulsified zone)}} \times 100
\]

Surface tensions measurement

The surface tension of extracted supernatant obtained from SSF process was measured by Krüss-K6 tensiometer, according to Velioglu and Urek, 2015. To increase the accuracy, the values were repeatedly taken thrice and average value was used to express the surface tension of the sample.

Biosurfactant production

The biosurfactant production under SSF was expressed as mg/gds. Each experiment was conducted in three replicates and average values are recorded.

3. Result and Discussion

The strain Serratia rubidaea SNAU02 was isolated from the hydrocarbon contaminated soils of Cuddalore district, Tamilnadu, India. The characterization of morphological and biochemical characters of the isolate Serratia rubidaea SNAU02 was carried out according to the Berge’s Manual of Determinative Bacteriology and the 16S rRNA sequencing was examined to determine the precise taxonomic position of the strain and identified as Serratia rubidaea. In the present study, the biosurfactant produced by Serratia rubidaea SNAU 02 was extracted using acid precipitation method. The characterization of biosurfactant confirmed the presence of Rhamnolipid biosurfactant from our previous findings (Nalini and Parthasarathi, 2013) and selected to the study the effect of agro-industrial by products as substrate for the production of biosurfactant by Serratia rubidae SNAU02 under SSF. The chemical composition of various agro-industrials by products/wastes was present in Table - 1.
Effect of agro-industrial by-products/wastes on emulsification index (% EI$_{24}$)

Emulsification index is one of the criteria to determine the potential of biosurfactant. Emulsifying index (% EI$_{24}$) determined the productivity of bioemulsifier (Bonilla et al., 2005). Emulsification execute when the surface tension reduction and reduced interface between oil and water provided excellent properties in terms of reduction of surface tension (Singh et al., 2007). Emulsification activity gave indication on the presence of biosurfactant. Higher emulsification index indicated a higher emulsification activity of the tested biosurfactant. To study the effect of various agro industrial by-products on emulsification index different concentration (4, 6, 8, 10 and 12 g) were used and inoculated with 2.5 ml of SNAU02. The different levels of oil cakes as substrate significantly influenced the emulsification index (Table - 2). Among the levels used, 8g of mahua oil cake recorded the higher emulsification index of 73 ± 0.86 % followed by peanut oil cake with emulsification index of 69 ± 0.12 %. This was followed by when 10 g of MOC which recorded the emulsification index of 65 ± 0.47 % followed by 63 ± 0.48 % of emulsification index with POC when used as substrate. According to Cooper and Goldenberg (1987), the emulsifying activity determines the strength of biosurfactant in retaining the emulsion of hydrocarbons or oils in water. The isolate SNAU02 showed MOC as the best substrate among the various substrates utilized in the biosurfactant production.

Effect of agro-industrial by-products/wastes on surface tension (mN/m)

The measurement of surface tension has traditionally been used to detect biosurfactant production and most of the other methods measure the surface properties of biosurfactant. Hence, the surface tension reduction method had been used by various earlier researchers (Willumensen and Karlson, 1997; Makkar and Cameotra, 1998 &1999). The agro industrial by products oil cake when used at different concentration (4, 6, 8, 10 & 12 g) and inoculated with SNAU02 significantly influenced the surface tension (Table - 3). Among the levels used, 8 g of MOC cake recorded the reduction of surface tension of 34 ± 0.21 mN/m. This was followed by 10 g of MOC utilized as substrate which registered the surface tension of 38 ± 0.65 mN/m. The POC registered second best substrate, surface tension of 35 ± 0.12 mN/m followed by SOC. Similar results were found in accordance with Jadhav et al. (2011).

Effect of agro-industrial by-products/wastes on biosurfactant production (mg/gds)

The agro industrial waste used at different concentration (4, 6, 8, 10 and 12 g) and inoculated with SNAU 02 significantly influenced the biosurfactant production. Among the levels used 8 g of MOC recorded the higher biosurfactant production of 81 ± 0.24 mg/gds. This was followed by 10 g of MOC as substrate with the biosurfactant production of 68 ± 0.24 mg/gds. Jain et al. (2013) reported alkaliphilic bacterium, Klebsiella sp. strain RJ-03 utilized different unconventional carbon sources such as mahua oil cake (Madhuca indica) for the production of biosurfactant due to its rich organic nature content (total sugars 73 %, reducing sugars 62.7 % and protein 8.6 %) proving them to be the most promising unconventional carbon for biosurfactant production. Peanut oil cake is a by-product obtained during the peanut oil manufacturing, used as animal feed which is rich in carbohydrate, protein and lipids. Thavasi et al. (2008) reported the biosurfactant production by using relatively cheap and abundantly available resources such as peanut oil cake and waste motor lubricant oil as a substrate, achieved a better yield for the biosurfactant production. The second best biosurfactant production was seen when peanut oil cake was used as the substrate. Next best source of substrate for biosurfactant production was found to be peanut oil cake followed by, SOC, CSOC, COC and GOC and respectively. The 8 g of POC registered the 71 ± 0.24 production of biosurfactant which was followed by 45 ± 0.24 of biosurfactant when 10 g of POC was utilized. The least production was obtained at 4 g of substrate. All the substrates studied for biosurfactant revealed that 8 % of substrate was found highly
Table 1: Chemical composition of various oil cakes.

<table>
<thead>
<tr>
<th>Agro industrial wastes</th>
<th>Carbohydrates (%)</th>
<th>Total fat (%)</th>
<th>Crude Protein (%)</th>
<th>Crude Fiber (%)</th>
<th>Ash content (%)</th>
<th>Nitrogen (%)</th>
<th>Phosphorus (%)</th>
<th>Potassium (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coconut oil cake</td>
<td>22±0.1</td>
<td>18±0.5</td>
<td>25±0.1</td>
<td>10.3±0.2</td>
<td>6.0±0.3</td>
<td>4.0±0.2</td>
<td>1.1±0.2</td>
<td>10±0.5</td>
</tr>
<tr>
<td>Castor oil cake</td>
<td>24±0.2</td>
<td>19±0.2</td>
<td>24.3±0.1</td>
<td>2.4±0.4</td>
<td>3.2±0.9</td>
<td>4.0±2.0</td>
<td>1.0±0.4</td>
<td>10±0.3</td>
</tr>
<tr>
<td>Gingelly oil cake</td>
<td>25±0.4</td>
<td>13±0.1</td>
<td>30±0.2</td>
<td>7.8±0.1</td>
<td>11.8±0.6</td>
<td>5.0±2.0</td>
<td>1.1±0.1</td>
<td>6.8±0.4</td>
</tr>
<tr>
<td>Peanut oil cake</td>
<td>30±0.3</td>
<td>21±0.4</td>
<td>24±0.3</td>
<td>5.3±0.2</td>
<td>4.5±0.2</td>
<td>4.0±0.5</td>
<td>0.74±0.1</td>
<td>10.0±0.1</td>
</tr>
<tr>
<td>Mahua oil cake</td>
<td>53±0.2</td>
<td>7±0.2</td>
<td>16±0.4</td>
<td>6.4±0.1</td>
<td>6.0±0.8</td>
<td>2.6±0.4</td>
<td>0.5±0.1</td>
<td>6.5±0.2</td>
</tr>
<tr>
<td>Sunflower oil cake</td>
<td>30±0.1</td>
<td>19±0.1</td>
<td>34±0.2</td>
<td>13±0.2</td>
<td>6.6±0.9</td>
<td>7.0±0.1</td>
<td>1.3±0.1</td>
<td>7.2±0.2</td>
</tr>
</tbody>
</table>

Table 2: Effect of various agro-industrial by-products/wastes on emulsification index (%EI$_{24}$)

<table>
<thead>
<tr>
<th>Levels (g)</th>
<th>Peanut oil cake(POC)</th>
<th>Coconut oil cake(COC)</th>
<th>Sunflower oil cake (SOC)</th>
<th>Castor oil cake (CSOC)</th>
<th>Gingelly oil cake(GOC)</th>
<th>Mahua oil cake(MOC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>57±0.15$^d$</td>
<td>48±0.95$^c$</td>
<td>47±0.45$^e$</td>
<td>55±0.25$^b$</td>
<td>54±0.54$^c$</td>
<td>58±0.24$^d$</td>
</tr>
<tr>
<td>6</td>
<td>61±0.24$^c$</td>
<td>43±0.53$^d$</td>
<td>51±0.45$^d$</td>
<td>54±0.47$^e$</td>
<td>52±0.57$^d$</td>
<td>62±0.54$^e$</td>
</tr>
<tr>
<td>8</td>
<td>69±0.12$^a$</td>
<td>58±0.72$^b$</td>
<td>65±0.95$^b$</td>
<td>58±0.15$^a$</td>
<td>57±0.25$^a$</td>
<td>73±0.86$^a$</td>
</tr>
<tr>
<td>10</td>
<td>63±0.48$^b$</td>
<td>50±0.35$^a$</td>
<td>63±0.64$^a$</td>
<td>43±0.85$^d$</td>
<td>56±0.35$^b$</td>
<td>65±0.47$^b$</td>
</tr>
<tr>
<td>12</td>
<td>48±0.91$^e$</td>
<td>41±0.12$^e$</td>
<td>49±0.85$^c$</td>
<td>40±0.47$^e$</td>
<td>47±0.47$^c$</td>
<td>58±0.10$^d$</td>
</tr>
</tbody>
</table>

All the values are mean ± SD for triplicate determinations. Different letters in the same column indicates significant differences (p<0.05). Within column different letter after values indicate that there is a significant difference at a P value of 0.05 as determined by DMRT.
### Table – 3: Effect of various agro-industrial by-products/ wastes on surface tension (mN/m)

<table>
<thead>
<tr>
<th>Levels (g)</th>
<th>Peanut oil cake(POC)</th>
<th>Coconut oil Cake(COC)</th>
<th>Sunflower oil cake (SOC)</th>
<th>Castor oil Cake (CSOC)</th>
<th>Gingelly oil Cake(GOC)</th>
<th>Mahua oil Cake(MOC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>45±0.41 c</td>
<td>42±0.76 a</td>
<td>46±0.12 c</td>
<td>45±0.12 c</td>
<td>43±0.15 b</td>
<td>45±0.12 b</td>
</tr>
<tr>
<td>6</td>
<td>41±0.25 d</td>
<td>40±0.35 b</td>
<td>41±0.25 d</td>
<td>43±0.45 a</td>
<td>41±0.25 c</td>
<td>40±0.45 a</td>
</tr>
<tr>
<td>8</td>
<td>35±0.12 e</td>
<td>38±0.45 d</td>
<td>36±0.10 c</td>
<td>37±0.12 e</td>
<td>38±0.76 d</td>
<td>34±0.21 c</td>
</tr>
<tr>
<td>10</td>
<td>40±0.14 b</td>
<td>41±0.68 c</td>
<td>47±0.58 b</td>
<td>56±0.45 a</td>
<td>42±0.45 e</td>
<td>38±0.65 a</td>
</tr>
<tr>
<td>12</td>
<td>52±0.15 a</td>
<td>44±0.14 a</td>
<td>51±0.43 a</td>
<td>54±0.54 b</td>
<td>46±0.98 a</td>
<td>43±0.48 c</td>
</tr>
</tbody>
</table>

All the values are mean ± SD for triplicate determinations. Different letters in the same column indicates significant differences (p<0.05). Within column different letter after values indicate that there is a significant difference at a P value of 0.05 as determined by DMRT.

### Table - 4: Effect of various agro-industrial by-products/ wastes on biosurfactant production (mg /gds)

<table>
<thead>
<tr>
<th>Levels (g)</th>
<th>Peanut oil cake(POC)</th>
<th>Coconut oil Cake(COC)</th>
<th>Sunflower oil cake (SOC)</th>
<th>Castor oil Cake (CSOC)</th>
<th>Gingelly oil Cake(GOC)</th>
<th>Mahua oil Cake(MOC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>32±0.25 d</td>
<td>26±0.65 e</td>
<td>31±0.12 c</td>
<td>36±0.91 c</td>
<td>36±0.48 c</td>
<td>31±0.68 e</td>
</tr>
<tr>
<td>6</td>
<td>54±0.48 b</td>
<td>35±0.58 c</td>
<td>42±0.45 c</td>
<td>34±0.10 d</td>
<td>24±0.63 d</td>
<td>57±0.48 c</td>
</tr>
<tr>
<td>8</td>
<td>71±0.15 a</td>
<td>40±0.87 b</td>
<td>55±0.75 a</td>
<td>52±0.21 a</td>
<td>39±0.10 a</td>
<td>81±0.24 a</td>
</tr>
<tr>
<td>10</td>
<td>45±0.41 c</td>
<td>38±0.83 a</td>
<td>48±0.15 b</td>
<td>45±0.54 b</td>
<td>38±0.48 b</td>
<td>68±0.58 b</td>
</tr>
<tr>
<td>12</td>
<td>28±0.24 e</td>
<td>24±0.48 d</td>
<td>24±0.68 d</td>
<td>16±0.36 c</td>
<td>22±0.96 e</td>
<td>43±0.47 d</td>
</tr>
</tbody>
</table>

*gds- gram dry substrate

All the values are mean ± SD for triplicate determinations. Different letters in the same column indicates significant differences (p<0.05). Within column different letter after values indicate that there is a significant difference at a P value of 0.05 as determined by DMRT.

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suitable for biosurfactant production followed by 10 %. The use of agro-industrial residues and by-products as feedstock in SSF processes on one hand adds economic value to these wastes or by-products, and on other hand its solve the problem of their disposal, which otherwise would cause pollution (Nigam et al., 2009).

4. Conclusion

The various agro-industrial by products screened showed higher production of biosurfactant using S. rubidaea SNAU02 under SSF when mahua oil cake was used as the substrate when compared with other substrates. All the substrates studied for biosurfactant production revealed that 8 % of substrate was found highly suitable for production of biosurfactant. These results highly encourage choosing easily available and cost effective substrates for biosurfactant production.

5. References